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Botulism results from the action of a protein neurotoxin (MW = 150,000) produced by the bacterium Clostridium botulinum, of which there are eight known strains. Botulinum neurotoxin is the most potent biological toxin known, having a median lethal dose of 5-50 ng/kg body weight. The primary site of action of botulinum toxin is the cholinergic nerve terminal, where it blocks the release of the neurotransmitter acetylcholine. Death usually results from respiratory failure. Nonlethal doses of botulinum toxin can induce sprouting of the nerve terminal and have significant postsynaptic effects, including muscle atrophy (continued)

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THE ACTION OF BOTULINUM TOXIN AT THE NEUROMUSCULAR JUNCTION

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Running Title: Action of botulinum toxin


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JUNCTION; ACETYLCHOLINE; TRANSMITTER RELEASE; PARALYSIS

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ABSTRACT

Botulism results from the action of a protein neurotoxin (MW = 150,000) produced by the bacterium Clostridium botulinum, of which there are eight known strains. Botulinum neurotoxin is the most potent biological toxin known, having a median lethal dose of 5-50 ng/kg body weight. The primary site of action of botulinum toxin is the cholinergic nerve terminal, where it blocks the release of the neurotransmitter acetylcholine. Death usually results from respiratory failure. Nonlethal doses of botulinum toxin can induce sprouting of the nerve terminal and have significant postsynaptic effects, including muscle atrophy and alterations in the membrane electrical properties of the muscle fiber. There is no universally available treatment for botulinum intoxication. However, immunotherapeutic and chemotherapeutic procedures are now being developed and will be discussed.

INTRODUCTION

Clostridium botulinum, the organism responsible for botulism, is an anaerobic, spore-forming, and rod-shaped bacterium. Cases of human botulism are often classified in one of the following categories: food poisoning, wound botulism, or infant botulism (78). The most commonly known form of botulism occurs after ingestion of food contaminated with the organism and preformed toxin. Wound botulism, the rarest form, results from infection at the wound site with subsequent toxin production and absorption. Infant botulism occurs during intraintestinal growth of the bacterium and may be a factor related to sudden infant death syndrome (3). Clinical symptoms arise from the action of a neurotoxin which is synthesized and released by the organism. The primary site of action of the neurotoxin is at cholinergic nerve terminals where it inhibits release of the neurotransmitter, acetylcholine (1, 2, 14-16, 18, 32, 57, 80). The blockade is most prominent in cranial nerves, autonomic nerves, and at the neuromuscular junction, which accounts for the clinical manifestations of diplopia, dysphagia, and dysarthria (78). The cause of death is usually respiratory paralysis due to the blockade of transmitter release from the phrenic nerve to the diaphragm muscles. Most of our present knowledge concerning the mode of action and the physiological consequences of the botulinal neurotoxins have resulted from studies of its effects on the neuromuscular junction. Therefore, this review will restrict itself to a discussion of that topic. Details concerning other aspects of the toxin, the organism, or the disease process can be found elsewhere (76, 78).

THE NEUROTOXINS

The neurotoxins of C. botulinum are found in at least eight immunologically distinct types: A, B, C₁, C₂, D, E, F, and G (73). In most instances an individual strain of C. botulinum will produce only one type of toxin (73). The toxins are synthesized during cell growth (10, 12) as protoxins or slightly toxic progenitor toxins (11, 52) and released by cell lysis (76). The method by which each toxin type attains full toxicity varies. However, three basic mechanisms appear to be common: (a) action of an endogenous protease on the toxin precursor, (b) action of an exogenous protease on a slightly active toxin precursor, or (c) synthesis of a fully active toxin requiring no activating protease. For example, type A, and some proteolytic strains of types B and F, produce a protease which activates the toxin precursor (20). The toxins produced by most types E and G strains, and some of their nonproteolytic strains, must be activated by an exogenous protease, e.g., trypsin (39). In contrast, some type E toxins are synthesized in a fully active form (39).

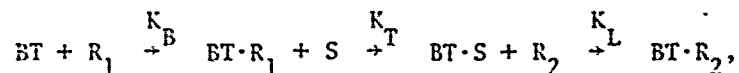
The first botulinal toxin to be crystallized was type A (51), which was shown to have a molecular weight of about 900,000 daltons (46, 68). It soon became clear, however, that the crystallized substance was composed of two components because the crystals also possessed hemagglutinating properties, which did not affect toxicity (50). These results suggested that the crystalline toxin had two distinct subunits, a neurotoxin and a hemagglutinating factor. Separation of these components was accomplished with ultracentrifugation of crystalline solutions at various alkaline pHs and ionic strengths (86, 87). Utilizing ion exchange chromatography, Das Gupta et al. (19) successfully separated the crystalline toxin into its neurotoxin (alpha fraction) and its

hemagglutinating components (beta fraction). The neurotoxin was shown to have a molecular weight of 150,000 daltons, while the hemagglutinin fragment was 500,000 daltons^{*}. The neurotoxins from types A, B, C, D, E, and F were found to have molecular weights of 150,000 (19), 167,000 (6), 141,000 (79), 170,000 (62), 135,000 (47), and 150,000 (88) daltons, respectively. The molecular weight of the type G neurotoxin has yet to be established.

The neurotoxin itself can be fragmented by disulfide bond reduction, yielding two nontoxic subunits (6, 48). It is interesting to note that the subunits from neurotoxins A (53,000 + 97,000), B (59,000 + 104,000), D (60,000 + 110,000), E (50,000 + 102,000) and F (56,000 + 105,000) have approximately the same molecular weights (6, 20, 62, 88). Although almost all the studies on the effects of botulinal neurotoxin have been done with type A, it is believed that all the toxin types exert their effect by the same mechanism.

UPTAKE OF TOXIN

Much of the work relating to the uptake kinetics of the botulinal neurotoxins has been carried out by Simpson (70-72, 74). Recently he presented a sequence for toxin uptake which includes: binding, translocation, and lytic steps (74). This sequence was described by Simpson (74) with the following equation:



where the toxin (BT) binds to a receptor (R_1) on the external surface of

* Measurement of molecular weight of large proteins is subject to an error factor of up to 20%.

the nerve terminal with a reaction rate K_B . A translocation step (K_T) involves movement of the toxin receptor complex ($BT \cdot R_1$) to a new site (S) within the nerve terminal. In the final step, the toxin complex ($BT \cdot S$) binds to an internal receptor site (R_2), where it blocks transmitter release.

In the binding step the toxin behaves like an irreversibly acting ligand. Since extensive tissue washing does not affect the activity of bound toxin, if dissociation of the toxin from the receptor occurs, the rate constant must be small. This step has a low Q_{10}^* (≈ 1.6), is not dependent on neuronal activity and is, therefore, probably not rate-limiting (74). During the binding step, the toxin is vulnerable to action by a type-specific antitoxin (immunoglobulin). This suggests that the binding moiety and the antigenic moiety are not identical. Furthermore, toxoid (inactivated toxin), although fully antigenic, does not itself bind to the nerve terminal membrane and, therefore, is unsuitable as a competitive inhibitor to the neurotoxins. Unlike some other presynaptic toxins, such as β -bungarotoxin, taipoxin and notexin, the botulinal neurotoxins do not have calcium-dependent phospholipase A_2 activity, thereby eliminating this specific enzymatic reaction as a mechanism of action in the binding step (74). The identity of the toxin receptor(s) for all eight toxin types is unknown.

The necessity for a translocation step is based on the observation that the access of antitoxin to the bound toxin does not decrease proportionally with the rate of onset of paralysis (74). This suggests an intermediate step between a binding step and a paralytic step.

*The alteration in the magnitude of any measurable parameter as a function of a 10°C change in temperature.

Translocation of the toxin into the nerve terminal is dependent on nerve activity (74). After binding is complete, the toxin may be taken up by endocytosis of nerve terminal membrane (81) during the synaptic vesicle recycling process (36) subsequent to nerve stimulation. The uptake of bound toxin with the recycled vesicle membrane could explain why nerve activity shortens the time required for the onset of paralysis (38), and how a molecule of 150,000 molecular weight can be taken up so readily (81).

The final lytic step, which may involve more than one step (74) leads to the actual blockade of transmitter release. This step is unaffected by antitoxin, has a high Q_{10} (≈ 4), and is dependent on the process of transmitter release (74). The lytic step may be the rate-limiting step in this sequence. It is unknown, whether the toxin molecule moves freely within the nerve terminal to the site of blockade or is transported there while associated with a synaptic vesicle. The precise site of blockade also is unknown, but it is presumed to be the "active zone" region (17), where most transmitter release is thought to occur (37). A hypothetical scheme for the uptake of toxin at the nerve terminal is illustrated in Figure 1.

PRESYNAPTIC EFFECTS

Mechanism of blockade

Acetylcholine is synthesized in the motor nerve terminal (35, 67), packaged and released in vesicles as quantal units (21, 22, 28, 29) or released as individual molecules, commonly referred to as nonquantal or molecular release (42-44) (Fig. 1). Acetylcholine is normally released spontaneously or as a result of nerve stimulation. As the name implies, spontaneous release occurs randomly in both the quantal and the molecular

forms. However, molecular release was estimated to account for 98-99% of the total spontaneous release of acetylcholine under normal conditions (30, 44). In contrast, nerve-evoked release is predominantly quantal in nature, although one may assume that some molecular release also occurs after nerve stimulation. Quantal release is dependent on the entry of calcium into the nerve terminal (27, 42). The increase in intracellular calcium causes the vesicles containing acetylcholine to fuse (exocytosis) with specialized areas of the nerve terminal membrane called "active zones" (8, 17, 36, 37) and release their contents into the synaptic cleft to interact with the postsynaptic acetylcholine receptors.

Acetylcholine release can be reduced by blocking any one of the steps involved in synthesis, storage, or release of transmitter. However, botulinum toxin does not affect impulse conduction down the nerve or at the nerve terminal (34). Although completely paralyzed, muscles still respond normally to both direct stimulation or direct application of acetylcholine by intraarterial injection or micro-iontophoretic application (9, 32, 34). Botulinum toxin does not affect the synthesis and storage of acetylcholine, the number of synaptic vesicles or the ultrastructure of the nerve terminal (16, 41, 80). In addition, the toxin does not block calcium from entering the nerve terminal (18, 41). Based on these studies, it may be concluded that botulinum toxin exerts its effect by blocking some step after the excitation of the nerve terminal, apparently affecting the release process (exocytosis) itself (18, 41, 81, 83).

Spontaneous transmitter release

When quanta (synaptic vesicles) are released individually in a random fashion from the nerve terminal, the interaction of the

acetylcholine from these quanta with the postsynaptic membrane produces a local depolarization called miniature end-plate potentials (m.e.p.p.s).

Quantitative measurements of this spontaneous quantal release of acetylcholine can be made by the analysis of m.e.p.p.s recorded by means of an intracellular microelectrode inserted at the postsynaptic membrane, thereby demonstrating the degree of blockade due to toxin injection. Measurements of this type indicated that botulinum toxin causes a reduction in the frequency and amplitude of m.e.p.p.s in the period immediately after administration (13, 18, 34, 77, 85). However, the frequency and amplitude of m.e.p.p.s increased with time after toxin injection (Table 1). The amplitude distribution of m.e.p.p.s was never normal after administration of the toxin, initially containing a population of very small m.e.p.p.s, and thereafter containing a mixed population of small and large m.e.p.p.s (18). It is interesting to note however, that the amplitude distribution of m.e.p.p.s in poisoned muscles could be shifted toward a more normal distribution by using procedures which increase spontaneous transmitter release, e.g., Ca ionophore A23187 together with high Ca^{++} , addition of black widow spider venom or high frequency stimulation of the nerve (13, 18, 34, 77). These data support the premise that botulinum toxin acts presynaptically and does not affect the number of normal vesicles of acetylcholine, but it does affect the spontaneous release of these vesicles (18).

As mentioned earlier, acetylcholine is released from motor nerve terminals in vesicles as quantal units or as individual molecules. However, only a small fraction of spontaneous acetylcholine release is quantal in nature. Most of the acetylcholine released from the motor nerve terminal in the absence of nerve stimulation is in the form of molecular leakage. After botulinum toxin poisoning, m.e.p.p. frequency

is reduced to only a few percent of normal (18). Brooks (14) demonstrated that total release is reduced only by one third, thereby indicating that molecular release is partially blocked by botulinum toxin. In a more recent study, Polak, et al. (66) showed that spontaneous release of acetylcholine was reduced up to 60% of control value after poisoning with botulinum toxin. This reduction in transmitter release may have given rise to the increase in acetylcholine content inside nerve terminals treated with the toxin (66). In addition, the increase in spontaneous transmitter release, which normally occurs after increasing extracellular potassium concentration in vitro was drastically reduced in botulinum-treated nerves (66). These data demonstrate that botulinum toxin almost completely abolishes spontaneous quantal release and reduces molecular release up to 60%.

Nerve-evoked transmitter release

Nerve stimulation causes the simultaneous release of hundreds of synaptic vesicles (quanta) from the nerve terminal. The acetylcholine from these vesicles causes a depolarization of the postsynaptic membrane known as an end-plate potential (e.p.p.s.). Within a few days after treatment with botulinum toxin, e.p.p.s evoked by nerve stimulation were reduced in amplitude approaching the size of small m.e.p.p.s (13, 18). With time, end-plate potentials increased in size and were occasionally made up of more than one quantum. However, end-plate potentials remained subthreshold and did not produce a muscle contraction upon nerve stimulation. Low frequency (0.5 Hz) repetitive stimulation resulted in a high number of failures; i.e., some stimulation pulses failed to produce a postsynaptic response, although nerve terminal depolarization was present in each

stimulus (18). The variability in the postsynaptic response due to botulinum poisoning is illustrated in Figure 2. At stimulus frequencies greater than 5 Hz, the number of failures was reduced and prolonged facilitation was observed (13, 18). The data obtained from measuring e.p.p.s after botulinum toxin poisoning indicated that the nerve-evoked transmitter release remained quantal in nature and followed Poisson statistics (18), although the amount of transmitter released was drastically reduced.

The receptor to which the toxin binds to produce the blockade of transmitter release is not known. Resolution of this dilemma may prove difficult because little is known concerning the release process under normal conditions. Hanig and Lamanna (33) have suggested a physical blockade of the release sites which occurs at the moment the vesicles fuse to nerve terminal membrane and that a ganglioside or neuraminidase may be an integral part of the receptor complex. Specifically, the sialic acid residue of a ganglioside might be involved in the binding reaction (75). Furthermore, Lamanna (49) hypothesized that the toxin blocks only when an opening exists during nerve stimulation. However, Leander and Thesleff (53) showed that increasing the number of open release sites during the release process did not enhance the ability of the toxin to produce its blockade.

Cull-Candy et al. (18) concluded that the toxin acted by reducing the system's sensitivity to Ca, thereby increasing the intracellular concentration of Ca necessary for the excitation secretion process to proceed. This idea was based on experiments showing a restoration of transmitter release similar to normal muscle by methods which increase intracellular Ca levels, e.g., Ca ionophore with high extracellular Ca

concentration or prolongation of the nerve terminal action potential with tetraethylammonium (TEA) or tetanic stimulation.

Nerve sprouting

In addition to its effect on transmitter release, botulinum toxin induced sprouting of the motor nerve after local nonlethal injection (26). However, this effect occurred at different rates in "fast-twitch" (gastrocnemius) and "slow-twitch" (soleus) muscles (24). In soleus muscle, nerve sprouts were present in the muscle by 4 days after toxin injection (25) and their numbers increased progressively until the fifth to sixth week, when new end-plates were formed (24). In contrast, sprouting did not occur in the gastrocnemius muscle until 3 or 4 weeks after toxin injection and continued until the new end-plates were formed between 6 and 8 weeks (24, 25).

Sprouting apparently occurred to compensate for the lack of neuromuscular transmission at the original junction. The new nerve sprouts were associated with Schwann cells (25) and attempted to make contact in the area of the original end-plate region. The first nerve-muscle contacts were loose and irregular, often interrupted by the accompanying Schwann cells. Intricate postsynaptic folds, always seen at normal end-plates, were absent during the initial period of nerve-muscle contact. Normal end-plates were observed in the soleus muscle 4 months after toxin injection. However, many nerve terminals were scattered along the muscle fibers in areas without noticeable end-plates (25). In contrast to the soleus muscle, the gastrocnemius had some normal end-plates, while other end-plates had few and shallow postsynaptic folds when examined several weeks after toxin injection (25).

POSTSYNAPTIC EFFECTS

The postsynaptic action of botulinum toxin is indirect, producing "denervation-like" effects on the postsynaptic muscle fiber. This occurs through the toxin's ability to interfere with the neurotrophic effect which the nerve normally exerts on the muscle (23).

The first postsynaptic effect reported after toxin injection, in addition to paralysis, was muscle atrophy (32). This type of atrophy was shown to be similar in degree to that observed after surgical denervation (40). Between 2 weeks and 2 months after toxin injection, muscle weight and muscle fiber diameter decreased by 40%. The soleus muscle showed a decrease in total fiber number due to some degeneration. However, the nerves innervating the affected muscles were unchanged and the muscle spindles and intrafusal muscle fibers were not significantly affected by the toxin. It is interesting to note that, although fast-twitch and slow-twitch muscles were paralyzed at the same time after toxin injection, the soleus muscles became atrophic faster than the gastrocnemius (24).

Botulinum toxin also affects the distribution and characteristics of cholinesterase at the postsynaptic membrane. Cholinesterase activity has been observed in conjunction with nerve sprouts; however, this enzyme activity resulted from the nonspecific enzyme pseudo-cholinesterase rather than the true acetylcholinesterase (24). As new neuromuscular junctions were formed, the amount of pseudo-cholinesterase decreased (24).

In normal muscle only the end-plate, where receptors are present in high density, is sensitive to the depolarizing effect of acetylcholine (4, 84). Following denervation, however, the entire surface of the muscle fiber becomes sensitive to acetylcholine due to the insertion in

the membrane of new "extrajunctional" receptors (4, 84). Thesleff (80) showed that after the administration of botulinum toxin, the muscle became sensitive to applied acetylcholine to a similar degree and with approximately the same time course as denervated muscle. In a subsequent study (65), other investigators demonstrated that the number of extrajunctional acetylcholine receptors induced by botulinum toxin poisoning was somewhat less quantitative than after denervation. Since the toxin effect occurs without any apparent ultrastructural change in the nerve terminal, it is unlikely that the "denervation-like" effects are the result of nerve degeneration (80).

Both the administration of botulinum toxin and surgical denervation result in a depolarization of the resting membrane potential and the development of tetrodotoxin-resistant action potentials recorded from the muscle membrane (59). However, the extent of these effects was less in the toxin-treated muscles than in denervated muscles (59). This finding was similar to the observations made with respect to extrajunctional receptors.

In a preliminary report, Sellin and Thesleff (69) showed a change in the ion channel kinetics at the end-plate membrane after botulinum toxin poisoning, which were similar to the kinetics recorded from channels appearing in areas outside the end-plate after denervation (31). These observations suggest that botulinum toxin induces the appearance of new receptor-ion channel complexes at the end-plate and these complexes are similar to those observed in extrajunctional regions after denervation. In support of this hypothesis, Levitt et al. (54) showed that the turnover of acetylcholine receptors at the neuromuscular junction increases after denervation. They concluded from their data that the junction contains a dual population of receptors after denervation: the original receptors

present before denervation and new ones with turnover rates equivalent to extrajunctional receptors.

These data indicate that, even if transmitter release is restored, botulinum toxin may produce long-term alterations in the postsynaptic muscle membrane. These changes may be due to the ability of the toxin to interfere with a trophic substance of neuronal origin, which may be acetylcholine, nerve-induced muscle activity or some as yet unknown biochemical substance. It is clear, however, that the postsynaptic alterations due to botulinum toxin poisoning are similar in mode, but not degree, to those changes observed after surgical denervation.

PROPHYLAXIS AND THERAPY

Although there is no commercially available single product for the treatment of botulinum intoxication, research is in progress to establish procedures for prophylaxis and therapy. The prophylactic method involves immunization of individuals by stimulating antibody production after a series of three injections of a formalin-inactivated toxin (toxoid). There are two approaches for therapeutic intervention in nonimmunized individuals who have been exposed to the organism or the toxin. The first involves the use of specific antitoxin. The second method is by means of chemotherapeutic agents, whose mode of action would involve increasing transmitter release in nerve terminals already blocked by the toxin.

Immunization of animals with botulinal toxoids has been practiced for over 40 years (7). However, toxoids for human use were not available until the late fifties, when a polyvalent toxoid was developed to protect high-risk individuals against toxins A-E (61). This toxoid produced measurable quantities of toxin-neutralizing antibody when

injected into humans in a series of three initial injections over a 3-month period, with a booster after 12 months (61).

It is possible to use the antibody produced in immunized individuals as an antitoxin for individuals who have contracted botulism. However, the quantity of specific neutralizing antibody produced in persons immunized with botulinal toxoids during a single 12-month period of immunization is insufficient to justify its collection and use as a therapeutic agent (61). At present, the only licensed antiserum products available are those derived from horse serum (55), which produce side-reactions in 21% of recipients due to noncompatible proteins (60). Projects are under way to collect large quantities of human immune globulin by plasmapheresis of individuals who have received multiple doses of pentavalent (A-E) toxoid over a 5-year period (61)*. Such a program should produce a human immune globulin product of a low risk for the specific treatment of botulism.

There are two important problems in utilizing immunological techniques for botulinum poisoning. First, it is not feasible to immunize everyone against botulism, but only those in high-risk situations. Second, although antitoxin can be a useful therapeutic agent, it is only effective on circulating toxin or on toxin in the initial binding step. Antitoxin does not inhibit or reduce paralysis once the toxin is inside the nerve terminal. Therefore, agents which increase transmitter release are likely candidates for relieving the paralysis caused by botulinum toxin. The influx of Ca into the nerve terminal during stimulation is a prerequisite for transmitter release

* These individuals have high titers of antibody due to the long-term immunization.

during excitation-secretion coupling (42). Agents, such as the Ca ionophore A23187 and black widow spider venom, can increase spontaneous quantal release in botulinum-poisoned muscles (18, 57). Increasing extracellular Ca alone does not restore spontaneous quantal release to normal values (18). For nerve-evoked transmitter release, TEA and 4-aminopyridine increase quantal content in botulinum-poisoned muscles (18, 57, 58) (Fig. 3). Unfortunately, TEA has a curare-like action postsynaptically and, therefore, is less suitable than 4-aminopyridine as an antagonist of botulinum poisoning (58).

Aminopyridines, in particular 4-aminopyridine (4-AP) and 3,4-diaminopyridine (3,4-DAP), are known to stimulate the central nervous system, elevate blood pressure, and exert an anti-curare effect (82). These effects can be attributed to an increase in neural transmitter release (45, 56, 63, 64). It is presumed that 4-AP and 3,4-DAP increase Ca permeability by their blocking action of K efflux (rectification) during the nerve terminal action potential. The duration of the action potential is prolonged through this mechanism. A longer time in the depolarized state increases the amount of Ca which may enter the nerve terminal during excitation-secretion coupling. Since Ca ion is intimately involved in the transmitter secretion process, an increased transmitter release should result. The aminopyridine group of drugs are, therefore, promising antagonists to the paralytic effect of botulinum toxin, as demonstrated in animal experiments (58). During an outbreak of type E botulism in England, 4-AP was administered to four patients. These individuals showed a restoration of transmitter release (electromyograph) with an almost complete reversal of peripheral paralysis (5). However, the effect was transient and no effect was observed on the respiratory muscles.

It appears that a likely treatment for botulism might be the administration of a drug which increases transmitter release, like 4-AP or 3,4-DAP, plus an antitoxin. Separately, they both have drawbacks, but their combined effect should be synergistic. As mentioned previously, the antitoxin is only effective in neutralizing the toxin before binding or during the binding step, and is useless once the toxin enters the nerve terminal. Although aminopyridines are effective in increasing transmitter release even after the onset of paralysis, their stimulatory effect may actually increase the rate of toxin uptake during the early stages of the disease. Assuming toxin uptake is dependent on endocytotic uptake of nerve terminal membrane during the vesicle recycling process, aminopyridines alone would accelerate the rate of uptake of bound toxin. In combination, antitoxin could neutralize all circulating toxin or toxin in the binding step, while aminopyridine could antagonize the blocking effect of toxin inside the nerve terminal. While the proposed treatment is theoretical at this time, combination therapy warrants further consideration as a logical modality against botulinum intoxication.

REFERENCES

1. Ambache N: Peripheral action of botulinum toxin. *Nature* 161: 482-483, 1948
2. Ambache N: The peripheral action of Cl. botulinum toxin. *J Physiol* 108: 127-141, 1949
3. Arnon SS, Midura TF, Damus K, Wood RM, Chin J: Intestinal infection and toxin production by *Clostridium botulinum* as one cause of sudden infant death syndrome. *Lancet* 1: 1273-1277, 1978
4. Axelsson J, Thesleff S: A study of supersensitivity in denervated mammalian skeletal muscle. *J Physiol* 147: 178-193, 1959
5. Ball AP, Hopkinson RB, Farrell ID, Hutchinson JGP, Paul R, Watson RDS, Page AJF, Parker RGF, Edwards CW, Snow M, Scott DK, Leone-Canado A, Hastings A, Ghosh AC, Gilbert RJ: Human botulism caused by *Clostridium botulinum* type E: The Birmingham outbreak. *Q J Med* 48: 473-491, 1979
6. Beers WH, Reich E: Isolation and characterization of *Clostridium botulinum* type B toxin. *J Biol Chem* 244: 4473-4479, 1969
7. Bennetts HW, Hall HTE: Botulism of sheep and cattle in Western Australia: Its cause and prevention by immunization. *Aust Vet J* 14: 105-108, 1938
8. Birks R, Huxley HE, Katz B: The fine structure of the neuromuscular junction of the frog. *J Physiol* 150: 134-144, 1960
9. Bishop GH, Bronfenbrenner JJ: The site of action of botulinus toxin. *Am J Physiol* 117: 393-404, 1936
10. Bonventre PF, Kempe LL: Physiology of toxin production by *Clostridium botulinum* types A and B. IV. Activation of the toxin. *J Bacteriol* 79: 24-32, 1960

11. Bonventre PF, Lincoln RE, Lamanna C: Status of bacterial toxins and their nomenclature: Need for discipline and clarity of expression. *Bacteriol Rev* 31: 95-109, 1967
12. Boroff DA: Study of toxins of Clostridium botulinum. III. Relation of autolysis to toxin production. *J Bacteriol* 70: 363-367, 1955
13. Boroff DA, del Castillo J, Evoy WH, Steinhardt RA: Observations on the action of type A botulinum toxin on frog neuromuscular junctions. *J Physiol* 240: 227-253, 1974
14. Brooks VB: The action of botulinum toxin on motor-nerve filaments. *J Physiol* 123: 501-515, 1954
15. Brooks VB: An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. *J Physiol* 134: 264-277, 1956
16. Burgen ASV, Dickens F, Zatman LT: The action of botulinum toxin on the neuromuscular junction. *J Physiol* 109: 10-24, 1949
17. Couteaux R, Pécot-Dechaveassine M: Vésicules synaptiques et poches au niveau des "zones actives" de la jonction neuromusculaire. *C R Acad Sci [D] (Paris)* 271: 2346-2349, 1970
18. Cull-Candy SG, Lundh H, Thesleff S: Effects of botulinum toxin on neuromuscular transmission in the rat. *J Physiol* 260: 177-203, 1976
19. DasGupta BR, Boroff DA, Rothstein E: Chromatographic fractionation of crystalline toxin of Clostridium botulinum type A. *Biochem Biophys Res Commun* 22:750-756, 1966
20. Das Gupta BR, Sugiyama H: Role of a protease in natural activation of Clostridium botulinum neurotoxin. *Infect Immun* 6: 587-590, 1972
21. del Castillo J, Katz B: Quantal components of the end-plate potential. *J Physiol* 124: 560-573, 1954

22. De Robertis EDP, Bennett HS: Submicroscopic vesicular component in the synapse. Fed Proc 13: 35, 1954
23. Drachman DB: Trophic functions of the neuron. 3. Mechanisms of neurotrophic interactions. The role of acetylcholine as a neurotrophic transmitter. Ann NY Acad Sci 228: 160-176, 1974
24. Duchen LW: Changes in motor innervation and cholinesterase localization induced by botulinum toxin in skeletal muscle of the mouse: Differences between fast and slow muscles. J Neurol Neurosurg Psychiatr 33: 40-54, 1970
25. Duchen LW: An electron microscopic study of the changes induced by botulinum toxin in the motor end-plates of slow and fast skeletal muscle fibres of the mouse. J Neurol Sci 14: 47-60, 1971
26. Duchen LW, Strich SJ: The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. Q J Exp Physiol 53: 84-89, 1968
27. Elmqvist D, Feldman DS: Calcium dependence of spontaneous acetylcholine release at mammalian motor nerve terminals. J Physiol 181: 487-497, 1965
28. Elmqvist D, Quastel DMJ: Presynaptic action of hemicholinium at the neuromuscular junction. J Physiol 177: 463-482, 1965
29. Fatt P, Katz B: Spontaneous subthreshold activity at motor nerve endings. J Physiol 117: 109-128, 1952.
30. Fletcher P, Forrester T: The effect of curare on the release of acetylcholine from mammalian motor nerve terminals and an estimate of quantum content. J Physiol 251: 131-144, 1975
31. Cage PW, Hamill OP: Lifetime and conductance of acetylcholine-activated channels in normal and denervated toad sartorius muscle. J Physiol 298: 525-538, 1980

32. Guyton AC, MacDonald MA: Physiology of botulinus toxin. Arch Neurol Psychiatr 57: 578-592, 1947
33. Hanig JP, Lamanna C: Toxicity of botulinum toxin: A stoichiometric model for the locus of its extraordinary potency and persistence at the neuromuscular junction. J Theor Biol 77: 107-113, 1979
34. Harris AJ, Miledi R: The effect of type D botulinum toxin on frog neuromuscular junctions. J Physiol 217: 497-515, 1971
35. Hebb CO, Krnjević K, Silver A: Acetylcholine and choline acetyltransferase in the diaphragm of the rat. J Physiol 171: 504-513, 1964
36. Heuser JE, Reese JE: Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57: 315-344, 1973
37. Heuser JE, Reese TS, Dennis MJ, Jan Y, Jan L, Evans L: Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J Cell Biol 81: 275-300, 1979
38. Hughes R, Whaler BC: Influence of nerve-ending activity and of drugs on the rate of paralysis of rat diaphragm preparations by Cl. botulinum type A toxin. J Physiol 160: 221-233, 1962
39. Iida H: Activation of Clostridium botulinum toxin by trypsin. In: Toxic microorganisms, p. 336-340. Ed. M. Herzberg. U.S. Government Printing Office, Washington, D.C., 1970
40. Jirmanová I, Sobotková M, Thesleff S, Zelena J: Atrophy in skeletal muscles poisoned with botulism toxin. Physiol Bohemoslov 13: 467-472, 1964
41. Kao I, Drachman DE, Price DL: Botulinum toxin: Mechanism of presynaptic blockage. Science 193: 1256-1258, 1976

42. Katz B: The release of neural transmitter substances. Liverpool University Press, Liverpool, 1969
43. Katz B, Miledi R: The quantal release of transmitter substances. In: Studies in physiology, p. 118-125, Berlin, Springer 1965
44. Katz B, Miledi R: Transmitter leakage from motor nerve endings. Proc R Soc Lond (Biol) 196: 59-72, 1977
45. Katz B, Miledi R: Estimates of quantal content during "chemical potentiation" of transmitter release. Proc R Soc Lond Biol 205: 369-378, 1979
46. Kegeles G: The molecular size and shape of botulinus toxin. J Am Chem Soc 68: 1670, 1946
47. Kitamura H, Sakaguchi S, Sakaguchi C: Significance of the 12S toxin of Clostridium botulinum type E. J Bacteriol 98: 1173-1178, 1969
48. Kozaki S, Nivazaki S, Sakaguchi G: Development of antitoxin with each of two complementary fragments of Clostridium botulinum type B derivative toxin. Infect Immun 18: 761-766, 1977
49. Lamanna C: The pipe and valve hypothesis of the mechanism of the action of botulinical toxin. Ann Acad Sci Arts Bosnia Herzegovina Special Pub 29: 213, 1976
50. Lamanna C, Lowenthal JP: The lack of identity between hemagglutinin and the toxin of type A botulinical organism. J Bacteriol 61: 751-752, 1951
51. Lamanna C, McElroy OE, Eklund HW: The purification and crystallization of Clostridium botulinum type A toxin. Science 103: 613-614, 1946
52. Lamanna C, Sakaguchi G: Botulinical toxins and the problem of nomenclature of simple toxins. Bacteriol Rev 35: 242-249, 1971

53. Leander S, Thesleff S: On the mode of action of botulinum toxin.
Acta Physiol Scand 108: 195-196, 1980
54. Levitt TA, Loring RH, Salpeter JM: Neuronal control of acetylcholine receptor turnover rate at a vertebrate neuromuscular junction.
Science 210: 550-551, 1980
55. Lewis GE Jr, Metzger JF: Botulism immune plasma (human). Lancet 2: 634-635, 1978
56. Lundh H: Effects of 4-aminopyridine on statistical parameters of transmitter release at the neuromuscular junction. Acta Pharmacol Toxicol (Copenh) 44: 343-346, 1979
57. Lundh H, Cull-Candy SG, Leander S, Thesleff S: Restoration of transmitter release in botulinum-poisoned skeletal muscle. Brain Res 110: 194-198, 1976
58. Lundh H, Leander S, Thesleff S: Antagonism of the paralysis produced by botulinum toxin in the rat. The effects of tetraethylammonium, guanidine and 4-aminopyridine. J Neurol Sci 32: 29-43, 1977
59. Mathers DA, Thesleff S: Studies on neurotrophic regulation of murine skeletal muscle. J Physiol 282: 105-114, 1978
60. Merson MH, Hughes JM, Dowell VR, Taylor A, Barker WH, Gangarosa EJ: Current trends in botulism in the United States. JAMA 229: 1305-1308, 1974
61. Metzger JF, Lewis GE Jr: Human-derived immune globulins for the treatment of botulism. Rev Infect Dis 1: 689-690, 1979
62. Miyazaki S, Iwasaki M, Sakaguchi G: Clostridium botulinum type D toxin: Purification, molecular structure, and some immunological properties. Infect Immun 17: 395-401, 1977
63. Molgó I, Lemeignan M, Lechat P: Effects of 4-aminopyridine at the frog neuromuscular junction. J Pharmacol Exp Ther 203: 653-663, 1977

64. Molgó J, Lundh H, Thesleff S: Potency of 3,4-diaminopyridine and 4-aminopyridine on mammalian neuromuscular transmission and the effect of pH changes. Eur J Pharmacol 61: 25-34, 1980
65. Pestronk A, Drachman DB, Griffin JW: Effect of botulinum toxin on trophic regulation of acetylcholine receptors. Nature 264: 787-789, 1976
66. Polak RL, Sellin LC, Thesleff S: Acetylcholine content and release in botulinum-poisoned or denervated rat skeletal muscle. J Physiol (In press)
67. Potter LT: Synthesis, storage and release of [¹⁴C]acetylcholine in isolated rat diaphragm muscles. J Physiol 206: 145-166, 1970
68. Putnam FW, Lamanna C, Sharp DG: Molecular weight and homogeneity of crystalline botulinum A toxin. J Biol Chem 165: 735-736, 1946
69. Sellin LC, Thesleff S: Effect of botulinum toxin on end-plate currents in the rat. Neurosci Abstr 6: 779, 1980
70. Simpson LL: The interaction between divalent cations and botulinum toxin type A in the paralysis of the rat phrenic nerve-hemidiaphragm preparation. Neuropharmacology 12: 165-176, 1973
71. Simpson LL: Studies on the binding of botulinum toxin type A to the rat phrenic nerve-hemidiaphragm preparation. Neuropharmacology 13: 683-691, 1974
72. Simpson LL: Pharmacological studies on the subcellular site of action of botulinum toxin type A. J Pharmacol Exp Ther 206: 661-669, 1978
73. Simpson LL: Studies on the mechanism of action of botulinum toxin. In: Neurotoxins: Tools in neurobiology, Advances in cytoparmacology, Vol 3, p. 27-34. Eds. B. Ceccarelli and F. Clementi. Raven Press, New York 1979

74. Simpson LL: Kinetic studies on the interaction between botulinum toxin type A and the cholinergic neuromuscular junction. J Pharmacol Exp Ther 212: 16-21, 1980
75. Simpson LL, Rapport ME: Ganglioside inactivation of botulinum toxin. J Neurochem 18: 1341-1342, 1971
76. Smith L: Botulism. The organism, its toxin the disease. Charles C. Thomas Publisher, Springfield, Illinois, 1977
77. Spitzer N: Miniature end-plate potentials at mammalian neuromuscular junctions poisoned by botulinum toxin. Nature New Biol 237: 26-27, 1972
78. Sugiyama H: Clostridium botulinum neurotoxin. Microbiol Rev 44: 419-448, 1980
79. Svuto B, Kubo S: Isolation and molecular size of Clostridium botulinum type C toxin. Appl Environ Microbiol 33: 400-405, 1977
80. Thesleff S: Supersensitivity of skeletal muscle produced by botulinum toxin. J Physiol 151: 598-607, 1960
81. Thesleff S: Neurotoxins which block transmitter release from nerve terminals. Naunyn Schmiedebergs Arch Pharmacol 297: S5-S7, 1977
82. Thesleff S: Aminopyridines and synaptic transmission. Neuroscience 5: 1413-1419, 1980
83. Thesleff S, Lundh H: Mode of action of botulinum toxin and the effect of drug agonists. In: Neurotoxins: Tools in neurobiology, Advances in cytoparmacology, Vol. III. Eds. B. Ceccarelli and F. Clementi, p. 35-43, Raven Press, New York 1979
84. Thesleff S, Sellin LC: Denervation supersensitivity. Trends Neurosci 3: 122-126, 1980

85. Tonge DA: Chronic effects of botulinum toxin on neuromuscular transmission and sensitivity to acetylcholine in slow and fast skeletal muscle of the mouse. J Physiol 241: 127-139, 1974
86. Wagman J. Isolation and sedimentation study of low molecular weight forms of type A botulinus toxin. Arch Biochem 50: 104-112, 1954
87. Wagman J, Bateman JB: Botulinum type A toxin: Properties of a toxin dissociation product. Arch Biochem 45: 375-383, 1953
88. Yang KH, Sugiyama H: Purification and properties of Clostridium botulinum type F toxin. Appl Microbiol 29: 598-603, 1975

TABLE 1

Frequency and amplitude (mean and standard deviation) of miniature end-plate potentials recorded from rat extensor digitorum longus muscle*

Days after toxin injection	Frequency (sec ⁻¹)	Amplitude (mV)
Control	7.0 ± 1.47	0.6 ± 0.18
1	0.1 ± 0.06	0.3 ± 0.08
2	0.2 ± 0.11	0.4 ± 0.12
3-4	0.2 ± 0.06	0.6 ± 0.23
5-6	0.9 ± 0.43	1.3 ± 0.45
7-8	0.6 ± 0.16	1.5 ± 0.46
12-14	0.6 ± 0.11	2.4 ± 0.38

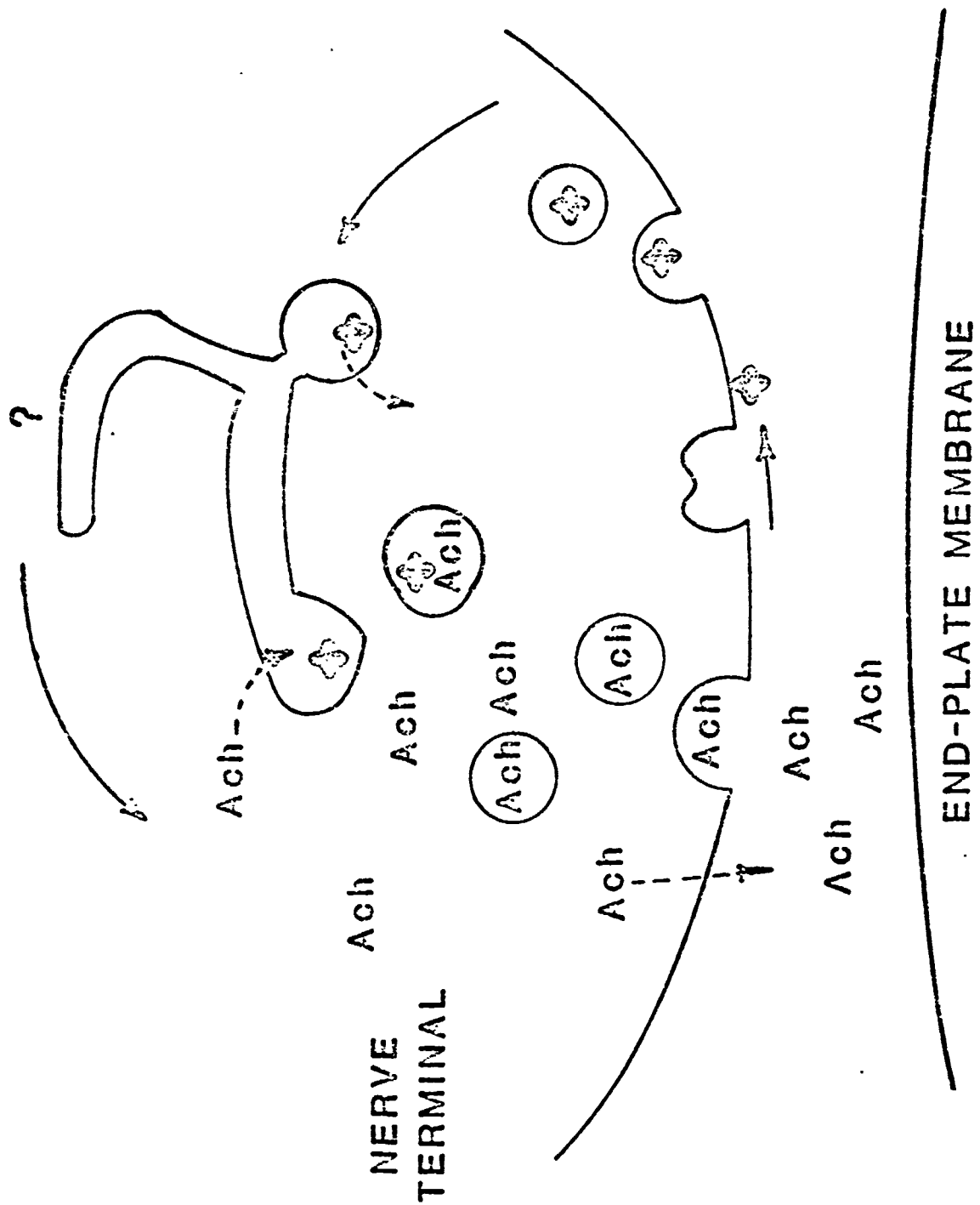
*Recorded at various times after local injection of botulinum toxin type A (= 5 mouse LD₅₀) (From Cull-Candy et al. 18).

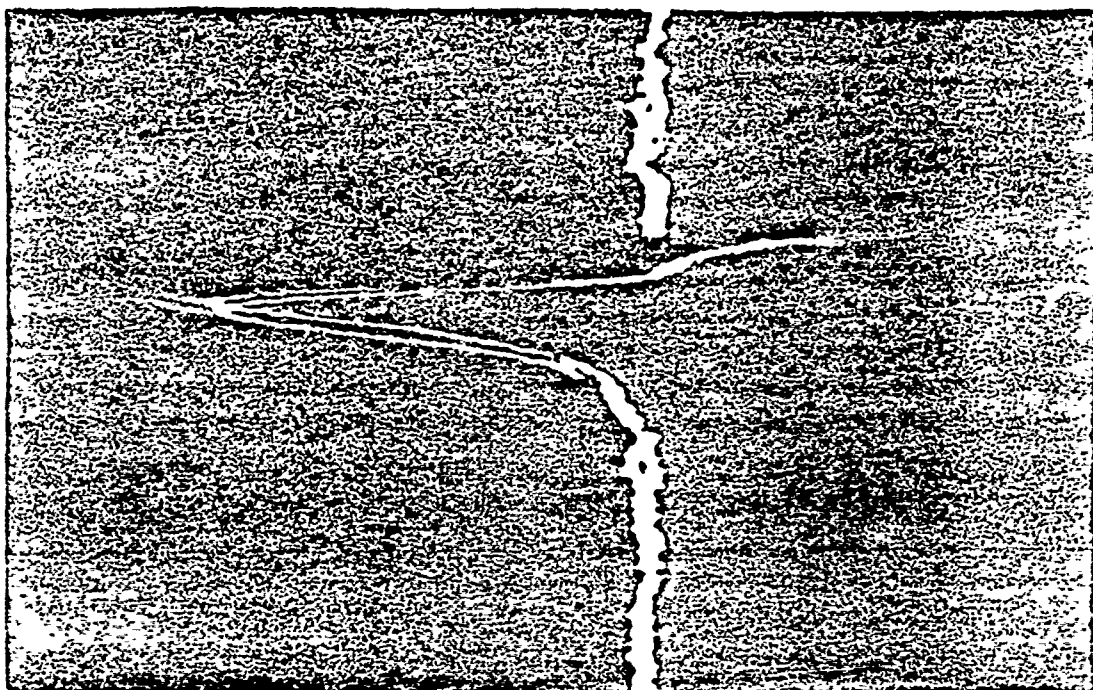
FIGURE LEGENDS

Fig. 1. Diagram showing the release of acetylcholine from the nerve terminal in molecular form (nonquantal) and within vesicles (quantal). Quantal transmitter release, whether spontaneous or evoked, is dependent on the influx of Ca for vesicle fusion to occur. After a vesicle fuses to the axolemma, membrane is recycled in a manner similar to that proposed by Heuser and Reese (36). Membrane is retrieved from the axolemma by endocytosis and coalesces to form cisternae which slowly divide to form new vesicles. It is not known how the new vesicles are refilled with acetylcholine. Botulinum toxin (as represented by the dark mass) binds to the membrane and may enter the nerve terminal via the recycled vesicles. It is not known (question mark) whether the toxin is released at some point in the recycling process to move freely to the blocking site or arrives at the site of blockage via a refilled vesicle [(adapted from Thesleff (81), Simpson (74), and Heuser and Reese (36))].

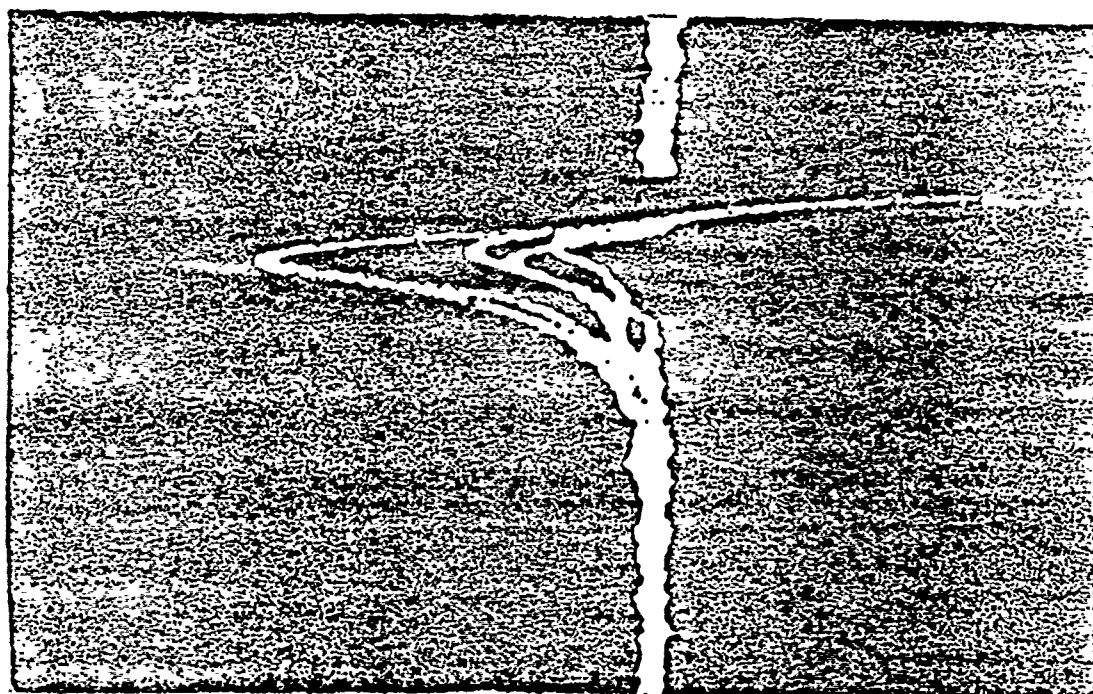
Fig. 2. End-plate currents recorded in vitro, after nerve stimulation, from rat extensor digitorum longus muscles (23°C) voltage clamped at -80 mV. Panel A shows end-plate currents from normal muscle whose release process was depressed by an extracellular magnesium concentration of 12 mM. However, the end-plate currents remained regular in amplitude. Panel B shows end-plate currents of variable amplitude from muscles two days after local injection of 5 mouse LD₅₀ botulinum toxin type A. In some experiments with botulinum-poisoned nerve-muscle preparations, nerve stimulation failed to produce a consistent postsynaptic response, or only a response equivalent to the release of a single quantum. The horizontal bar is 5 milliseconds and the vertical bar is 10 nanoamperes (Sellin and Thesleff, unpublished).

Fig. 3. Ability of 4-aminopyridine to increase transmitter release in botulinum-poisoned muscles (rat extensor digitorum longus). End-plate currents (e.p.c.s) as a function of clamped potential (mV) were recorded from normal (Δ) muscle, a muscle 7 days after injection of botulinum toxin (\bigcirc), and a botulinum-poisoned muscle (7 days) after the addition of 2 μ M 4-aminopyridine to the muscle bath (\square). Currents were recorded in vitro at 23°C; muscle contractions were prevented in normal muscle by using the crushed-fiber technique (69). 4-Aminopyridine clearly increased the postsynaptic response (e.p.c.) toward normal values at each clamped potential (Sellin and Thesleff, unpublished).





A



B

L

